Preparation of Superparamagnetic Immunomicrospheres and Application for Antibody Purification

Xianqiao Liu, Yueping Guan, Yu Yang, Zhiya Ma, Xiaobing Wu, Huizhou Liu

Laboratory of Separation Science and Engineering, State Key Laboratory of Biochemical Engineering, Institute of Process Engineering, Chinese Academy of Sciences, Beijing P.R. China
AGTC Gene Technology Company Ltd., Beijing, P.R. China

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ABSTRACT: A novel and effective protocol for the preparation of superparamagnetic immunomicrospheres has been developed. First, micro-size magnetic poly (methacrylate-divinylbenzene) (PMA-DVB) spheres were prepared by a modified suspension polymerization method. The oleic acid coated magnetite (Fe_3O_4) nanoparticles made by coprecipitation were mixed with monomers of MA, DVB, and initiator benzoyl peroxide (BPO) to form oil in water emulsion droplets with the presence of poly (vinyl alcohol) (PVA-1788) as a stabilizer. The polymerization reaction was carried out in a 2-L beaker equipped with four vertical stainless steel baffleplates by increasing the temperature of the mixture at a controlled rate. The resulting magnetic microspheres are micro-sized (less than 8μm in diameter) and 80 percent of them are in the size ranging from 1 to 5 μm. Then, they were highly functionalized via ammonolysis reaction with ethylenediamine, and the surface amino-modified magnetic microspheres were obtained. The morphology and properties of these magnetic microspheres were examined by SEM, TEM, VSM, and FT-IR. Affinity ligand protein A (ProtA) was covalently immobilized to the amino-modified magnetic microspheres by the glutaraldehyde method. These ProtA-immobilized magnetic immunomicrospheres were effective for affinity bioseparation processes, as was demonstrated by the efficient immunoaffinity purification of antibodies IgG2a (22mg per gram of microspheres) from mouse ascites. © 2004 Wiley Periodicals, Inc. J Appl Polym Sci 94: 2205–2211, 2004

Key words: synthesis; magnetic microspheres; copolymerization; antibody purification; bioengineering

INTRODUCTION

The application of magnetism to bioseparation has been actively investigated over the past few years.[1,2] This technology is based on the immobilization of an affinity ligand on the surface of prefabricated magnetic supports (microspheres, microbeads, and nanospheres) and the use of the resulting conjugates for the separation and concentration of biomolecules and cells.[3,4] Antibody-mediated isolation of cells is probably the best-known practical application of magnetic bioseparation owing to the speed, accuracy, and simplicity of the method.[5,6] These magnetic supports usually consist of inorganic magnetic core and polymeric shell that are either biocompatible or possessing active groups that can be conjugated to affinity ligands, such as protein A and streptavidin. The most important parameters of magnetic supports are size, structure, hydrophobicity/hydrophilicity, density of reactive surface groups, and superparamagnetic property.

Magnetic supports are often prepared via a monomer copolymerization method including suspension polymerization, emulsion polymerization, dispersion polymerization, and the two-step swelling method, which was originally developed by Ugelstad.[7] Polystyrene (PSt) and polymethyl methacrylate (PMMA) are the most frequently used polymers for magnetic microspheres. Methacrylic acid (MAA) and acrylic acid (AA) are often used as functional monomers.[8–10] However, the solubility of functional monomers is very limited (less than 10 wt % of monomers), and a large amount of functional groups were buried in the polymer with only a small part left on the surface. Among these methods, the suspension polymerization is simple and more suitable for massive production of spherical magnetic supports. However, it is hardly reported in literature that micro-size (several microns) magnetic spheres could be prepared by suspension polymerization. The magnetic polymer spheres made by conventional suspension polymerization are mostly in the size of several hundred micrometers with very broad size distribution.[11–13] They can be used as reactive supports and carriers after sieve, for fillings of chromatographic columns, and for enzyme

Correspondence to: H. Liu (hzliu@home.ipe.ac.cn).
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immobilization. The main disadvantages of these magnetic spheres are their large size, broad size distribution, and low density of surface reactive groups. In suspension polymerization, size and size distribution of droplets are controlled by the stirring condition and the concentration of the stabilizer. Therefore, the drawbacks of suspension polymerization can be overcome to a certain extent by improving the stirring condition and modifying the reaction process.

In this article, we describe a novel and effective process for the preparation of highly functionalized, micro-size superparamagnetic immunomicrospheres and their practical application for antibody purification from mouse ascites. Compared with other polymers, such as PSt-DVB and PMMA-DVB, PMA-DVB microspheres are much easier to be functionalized with surface ammonolysis to introduce reactive surface groups. Meanwhile, PMA-DVB are more hydrophilic so that the nonspecific adsorption could be avoided to some extent in the application of antibody purification. Therefore, MA was selected as the monomer herein. First, magnetic PMA-DVB microspheres were synthesized by the modified suspension polymerization of methacrylate and divinylbenzene in the presence of the magnetic fluid. Then the resulting magnetic microspheres were highly functionalized via ammonolysis reaction with ethylenediamine at the temperature of 110°C in the presence of dimethylformamide (DMF). The surface amino-modified magnetic functional microspheres were obtained by this process. The morphology, structure, magnetic property, and surface functionality of the magnetic microspheres were examined by SEM, TEM, VSM, and FT-IR. Protein A, as an affinity ligand, was covalently coupled to the surface of the amino-modified magnetic microspheres by the glutaraldehyde method. To test the effect of the magnetic microspheres, the ProtA-immobilized magnetic immunomicrospheres were successfully applied to the purification of antibody (IgG2a) from mouse ascites directly.

EXPERIMENTAL

Materials

Chemicals were generally of reagent grade from commercial sources. Methacrylate and divinyl benzene were distilled under reduced pressure to remove the inhibitor prior to use. Protein A (molecular weight 42kD) and mouse ascites were supplied by AGTC Gene Technology Company Ltd., Beijing, China. Protein solutions were buffered with 0.1*M* of sodium phosphate, pH 7.4. Low protein molecular mass marker was purchased from Sino-American Biotechnology Company, Beijing, China. All other materials were of analytical grade and used without any further purification, including ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), poly (vinyl alcohol) (PVA-1788), aqueous ammonia (25%[w/w]), oleic acid, benzoyl peroxide (BPO), ethylenediamine, dimethylformamide (DMF), glutaraldehyde, hexane, and ethanol.

Preparation of magnetic fluid

The magnetic fluid was made by the coprecipitation method. 23.4g $FeCl_3 \cdot 6H_2O$ and 8.6g $FeCl_2 \cdot 4H_2O$ were dissolved in 500 mL deionized water in a 2-L beaker under nitrogen gas with vigorous stirring at 85°C. 30 mL 25% $NH_3 \cdot H_2O$ was added to the solution. The 14 mL oleic acid was added dropwise into the suspension within 20 min. After several minutes, the magnetic precipitates were isolated from the solvent by magnetic decantation. The precipitates were washed with deionized water three times to remove the excess oleic acid. The magnetic precipitate was redispersed in an organic carrier liquid such as hexane to form the magnetic fluid.

Synthesis of magnetic PMA-DVB microspheres

Magnetic PMA-DVB microspheres were prepared by a modified suspension polymerization method.[14] 95 mL methacrylate was mixed with 5 mL DVB, 30g magnetic fluid, and 3.0g BPO to form the organic phase. 25g PVA-1788 was dissolved in 1000 mL deionized water. The above two mixtures were mixed together to form suspension and then transferred to a 2-L beaker equipped with four vertical stainless steel baffleplates, a condenser, a nitrogen inlet, and a 4-paddle stirrer. The mixture temperature was maintained at 45°C for 30 min and then increased to 60°C within 10 min. Finally, the temperature was increased to 70°C, and the reaction was carried out for 2 more hours with the stirring speed of 1000rpm. To remove an excess of stabilizer and other impurities, the resulting magnetic PMA-DVB microspheres were washed with ethanol and deionized water several times and isolated by magnetic decantation. The magnetic polymer microspheres are brown in color and exhibit magnetic properties.

Surface treatment and activation

5.0g magnetic PMA-DVB microspheres prepared above were redispersed in 100 mL DMF. Then 100 mL ethylenediamine was added. The mixture was reacted at 110°C with mild stirring for 12 h. The resulting surface amino-modified magnetic microspheres were thoroughly washed with deionized water. The presence of surface amino groups (—NH_2) was confirmed by FT-IR spectroscopy. To facilitate the covalent attachment of the affinity ligand, the amino groups on the surface of magnetic microspheres were activated

with 5%(V/V) glutaraldehyde solution in 0.1*M* saline phosphate buffer pH 7.4.[15,16] After agitating at 30°C for 12 h, the glutaraldehyde-activated magnetic microspheres were recovered by magnetic sedimentation. The resulting magnetic microspheres were then washed three times with deionized water and stored as a suspension.

Immobilization of Protein A onto magnetic microspheres

The magnetic immunomicrospheres were prepared by the covalent immobilization of protein A onto the surface of the amino-modified magnetic microspheres. 50mg glutaraldehyde-activated magnetic microspheres prepared above were transferred to a test tube and pipetted off the supernatant while leaving the microspheres undisturbed. Then, 5 mL protein A was added at pH 7.4. The mixture was incubated at room temperature for 4 h. Then the ProtA-immobilized magnetic microspheres were recovered by magnetic sedimentation and the supernatant retained for the residual protein concentration assay. The microsphere preparation was washed three times with deionized water and adsorption buffer (0.1*M* Na-phosphate buffer pH 8.1) to remove any unbound protein A, and the ProtA-immobilized magnetic immunomicrospheres were stored in the same buffer until further use.

Purification of antibodies using ProtA-immobilized magnetic immunomicrospheres

Monoclonal antibodies IgG2a were purified by the ProtA-immobilized magnetic immunomicrospheres from mouse ascites directly. In a typical experiment, 10mg ProtA-immobilized magnetic immunomicrospheres were added to a 1.5-mL eppendorf tube and washed twice with adsorption buffer by magnetic decantation. The washed magnetic immunomicrospheres were resuspended in 900 μL 0.1*M* Na-phosphate buffer pH 8.1 and then 100 μL mouse ascites were added. The mixture was incubated with slow tilt rotation mixing for 30 min at room temperature. The resulting magnetic microspheres were washed three times with adsorption buffer by magnetic decantation and the supernatant was removed. To elute the antibody IgG2a, the above magnetic microspheres were resuspended in 400 μL 0.1*M* citrate buffer pH 2.1 with slow rotation for 10 min. Then the microspheres were magnetically sedimented and the supernatant, containing purified IgG2a, was transferred to a clean eppendorf tube for antibody concentration assay. After this step, the supernatant obtained was concentrated with ethanol and freeze-dried.

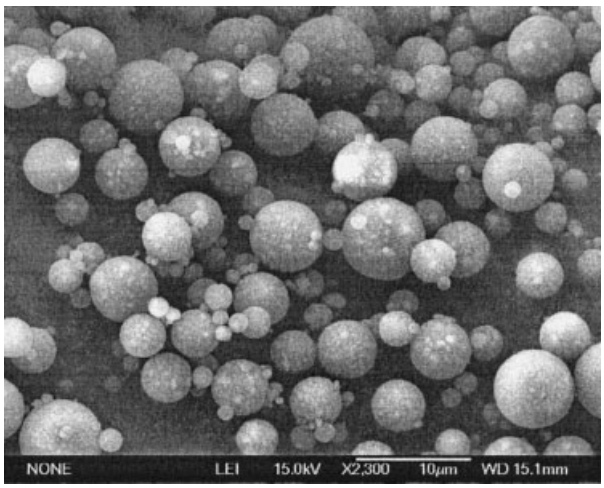


Figure 1 SEM of magnetic PMA-DVB microspheres.

Analysis and measurements

The morphology and structure of magnetic PMA-DVB microspheres were observed by scanning electron microscopy (SEM, JSM-6700F, JEOL, Japan) and transmission electron microscopy (TEM, H-8100, Hitachi, Japan). The magnetization curves of the samples were measured with a vibrating sample magnetometer (VSM, Model-155, Digital Measurement System, China). Surface functionality was characterized with the Fourier transform infrared spectroscopy (FT-IR, Vecter 22, Bruker, Germany) recorded using KBr pellets. The protein A and IgG2a antibody concentrations were determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) according to the manufacturer's manual. Sodium dodenylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 15% Tris-glycine gels using a Bio-Red mini gel apparatus. The 10 well, 0.75mm thick gels were run for 1.5 h at a constant 125V according to the manufacturer's instructions. The purified IgG2a and mouse ascites samples (10 μL) were first incubated with an equal volume of 2.5% 2-mercaptoethanol (loading buffer). The samples were then heated in a water bath at 80°C for 10 min, cooled, and spun at 13,000 rpm (Biofuge/Pico, Heraeus, Germany) before being applied to the gel. The gels were stained using Coomassie Brilliant Blue R.

RESULTS AND DISCUSSION

Synthesis of magnetic polymer microspheres

Micron-size magnetic PMA-DVB spheres were synthesized via a modified suspension polymerization method. The morphology and structure of the resulting microspheres were observed by SEM as shown in Figure 1 and with TEM in Figure 2. The particle size distributions were calculated by statistics from 300

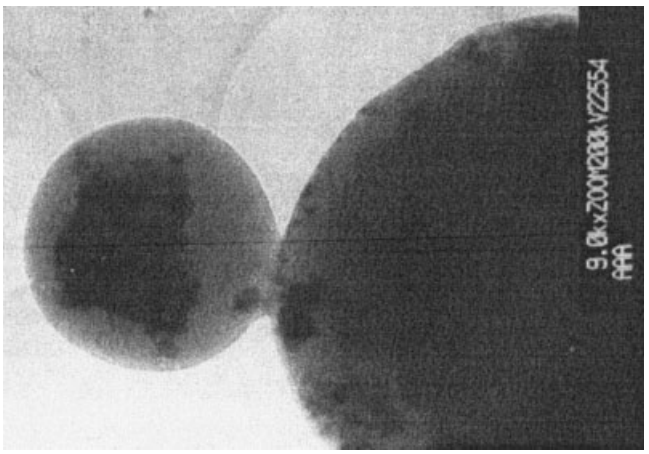


Figure 2 TEM of magnetic PMA-DVB microspheres.

particles in different regions of several SEM photos. Figure 3 shows the results. It can be seen that 80 percent of the particles are in the size ranging from 1 to 5μm. In suspension polymerization the initiator is soluble in the monomer phase, which is dispersed by agitation into the dispersion medium (usually water) to form droplets (i.e., an emulsion is formed). The combination of continued agitation and the addition of a suitable stabilizer (often a surface active polymer) have a stabilizing effect, hindering both the coalescence and further break-up of monomer droplets. The coalescence and break-up of monomer droplets will occur continuously during polymerization and the size distribution of droplets is usually very broad. Therefore, the particles made by the conventional suspension polymerization are most in the size range of several hundred micrometers with very broad size distribution.[11,13] In this study, nano-sized magnetite (Fe_3O_4) particles were obtained by the coprecipitation method from ferrous and ferric ion solution. They were in the average size of about 8nm and exhibited superparamagnetic characteristic. Then, these magnetite nanoparticles were coated with the oleic acid and became hydrophobic. Finally, they were mixed with monomers of MA, DVB, and initiator BPO to form oil in water emulsion droplets with the presence of poly (vinyl alcohol) (PVA-1788) as a stabilizer.

In theory, the size of the initial emulsion droplets formed is dependent upon the balance between droplet break-up and droplet coalescence. This is in turn controlled by the type and speed of agitator used, volume fraction of the monomer phase, and the type and concentration of stabilizer used. The stabilized monomer droplets may be considered as "microreactors," with the polymerization proceeding therein. This "mini-bulk" polymerization is initiated thermally and allowed to proceed to completion.[17] The average size of the polymer beads formed is expected to be approximately the same as that of the initial monomer droplets. Agitation results in the break-up of droplets. This will continue to occur until a stage in the reaction at which the partially polymerized beads are "sticky."[18] Control of coalescence and break-up rates is therefore critical for production of polymer beads of small and uniform size. The final bead size is slightly less than for the initial emulsion droplet size. In the present work, to control the size and size distribution of the droplets, two modifications were adopted. First, the polymerization was conducted in a 2-L beaker equipped with four vertical stainless steel baffleplates and a 4-paddle stirrer, so that the droplets dispersed were very uniform. Second, the reaction temperature was increased at a controlled rate with the increase in viscosity of droplets. It is important to control the process of temperature increase to ensure the period of droplet dispersion and the formation of small droplets. As expected, micron-size (less than 8μm in diameter) magnetic PMA-DVB spheres were prepared via this modified suspension polymerization method and 80 percent of them are in the size ranging from 1 to 5μm. On the contrary, if the temperature increases too fast as conventional suspension does, large (several hundred microns) and very nonuniform polymer spheres were obtained.

It is well known that an increase in the concentration of PVA increases the viscosity of the reaction medium and the amount of PVA adsorption on the particle surface. Consequently, the extent of droplet aggregation decreases, resulting in the decrease of the particle size. However, it is found that only a small amount of stabilizer added is adsorbed on the particles.[19] Therefore, it is necessary to add a large amount of stabilizer to obtain stable microspheres. This study shows that the size distribution becomes narrower if the magnetic microspheres are prepared with higher concentration of PVA (above 20 wt % of monomer) in

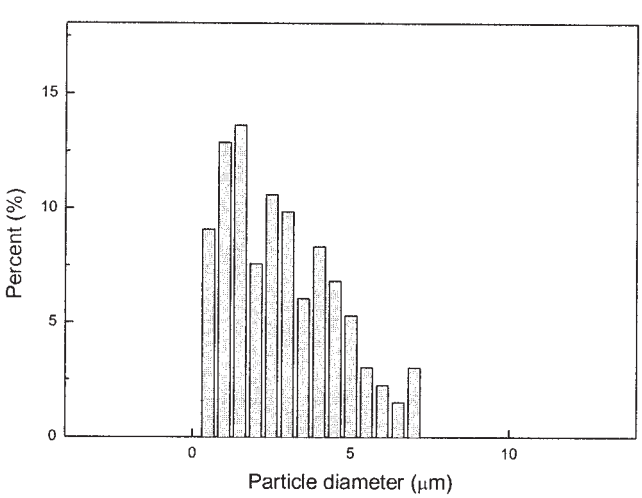


Figure 3 Size distribution of magnetic PMA-DVB microspheres.

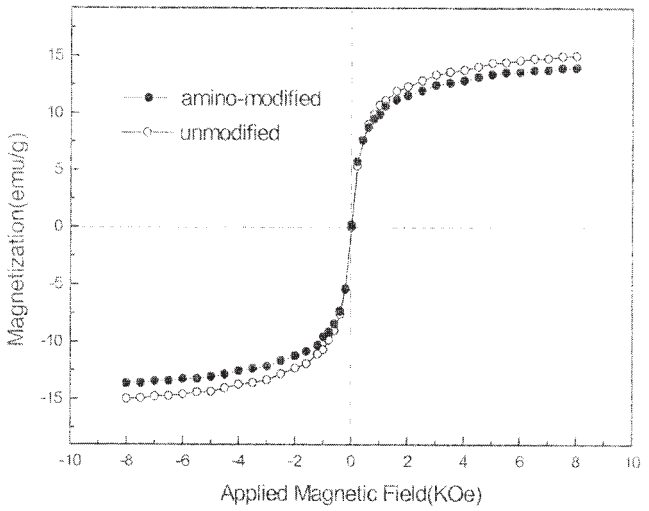


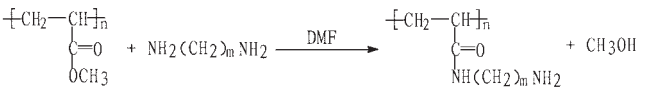
Figure 4 VSM magnetization curve of the amino-modified and unmodified magnetic PMA-DVB microspheres.

the system, which is much higher than that in the conventional suspension polymerization. It can be explained that the time taken for the stable particle formation stage becomes shorter with decrease of the nucleation time, resulting in the narrower size distribution of microspheres. However, after the polymerization is completed, the excessive stabilizer could only be washed away very slowly. In addition, at the very beginning of the polymerization reaction, free radicals are formed by initiator decomposition during the increase of temperature. Iron oxide in the polymerization mixture seemed to considerably decelerate the kinetics of the reaction because the magnetite is a strong inhibitor with adsorption of free radicals. Oxygen is also an inhibitor to the free radical polymerization. Therefore, a large amount of initiator BPO was added (3% wt of monomers) in this work.

The magnetic properties of the amino-modified and unmodified magnetic PMA-DVB microspheres were analyzed with VSM, as shown in Figure 4. The saturation magnetization of amino-modified microspheres, which was found to be 13.8 emu/g, is comparable to the unmodified microspheres of 15.0 emu/g. The result shows that the ammonolysis reaction process has little impact on the magnetism of microspheres before ammonolysis and after. It has been clear that, for ultrafine magnetically ordered particles, there exists a critical size below which the granules can acquire only single magnetic domains even in zero magnetic fields. The critical size was estimated at 25nm. This suggests that the magnetic microspheres prepared in this work are superparamagnetic with zero remanence and zero coercivity because the Fe_3O_4 particles used herein are about 8nm,[14,20] which are smaller than the Dp (superparamagnetic critical size) of Fe_3O_4 particles (Dp = 25nm). The magnetism of these magnetic microspheres preparation is higher than those of other similar works reported in the literature.[20–22] With such high saturation magnetization, both the amino-modified and unmodified magnetic microspheres can be easily separated within 20 s by a conventional permanent magnet (2000 Oersteds).

Surface functionality and ligand attachment

There are two principal difficulties in preparing high capacity magnetic microspheres for antibody purification. First, a significant reduction in particle size must be achieved to provide the surface area required, but too small a particle may not carry enough magnetite and, in practical terms, would cease to be magnetic. Second, an appropriate surface functionality should be introduced, with the density of functional groups on the microspheres being sufficiently high for efficient coupling of affinity ligands.[23] In this work, the magnetic PMA-DVB microspheres can be highly functionalized by ammonolysis reaction with ethylenediamine to form the amino-modified magnetic microspheres. The —OCH_3 groups on the surface of particles were replaced by ethylenediamine in the presence of DMF as follows.



The fact was proven by the comparison of FT-IR spectra of magnetic PMA-DVB microspheres before ammonolysis (A) and after (B), as shown in Figure 5. Before ammonolysis, the strong absorption bands at 1735 cm^{-1} indicate the existence of carboxylic ether due to C = O stretching vibration on the PMA-DVB microspheres. The C—O—C characteristic stretching vibration bands appear at 1160 cm^{-1} and 1270 cm^{-1}, as well as the —OCH_3 characteristic band at 1373

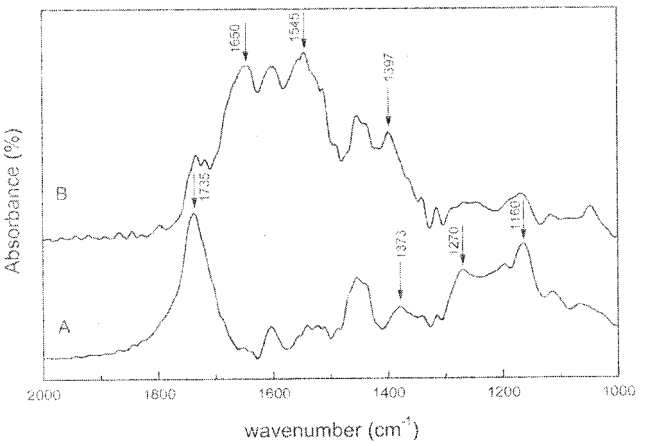


Figure 5 Comparison of FT-IR spectra of the magnetic PMA-DVB microspheres before ammonolysis (A) and after (B).

TABLE I
Immobilization of Protein A on Magnetic Microspheres*

Microsphere mass (mg)	Initial ProtA concentration (mg/ml)	Residual ProtA concentration in supernatant (mg/ml)	Immobilization capacity (mg/g)
50.0	0.5	0.26	24.0
50.0	1.0	0.75	25.0
50.0	2.0	1.75	25.0

* The immobilization of protein A onto the glutaraldehyde-activated magnetic microspheres was carried out in 5ml volume at pH 7.4 Na-phosphate buffer.

cm^{-1}. After ammonolysis, the intensity of the carboxyl band decreased due to the ammonolysis reaction that took place between the CH_3O—C = O groups and amino groups. The above —OCH_3 characteristic band at 1373 cm^{-1} disappeared. Meanwhile, the intensity of the C—O—C characteristic bands at 1160 cm^{-1} and 1270 cm^{-1} obviously decreased. It can also be seen that, compared with the unmodified magnetic microspheres, the amino-modified microspheres possess absorption bands at 1650 cm^{-1} and 1545 cm^{-1} due to the stretching vibration of the primary amino groups (—NH_2) and imide. All of these confirmed the ammonolysis reaction between ethylenediamine and PMA-DVB microspheres.

The amino-modified magnetic microspheres can be modified for a variety of biological applications. They are often transferred to aldehyde-group-containing magnetic microspheres by the glutaraldehyde method. For some biological applications, the aldehyde groups on the surface are more reactive groups and ready for immobilization of proteins or amino-group-containing affinity ligands. In this study, affinity ligand protein A was covalently immobilized onto the amino-modified magnetic microspheres by the glutaraldehyde method via the formation of Schiff base linkage with 5%(V/V) glutaraldehyde in Na-phosphate buffer pH 7.4.[16] As shown in Table I, the capacity of immobilization is about 25mg per gram of microspheres under the experimental conditions used, as judged by three independent tests. The ProtA-immobilized magnetic microspheres were then ready for the antibody purification.

Affinity purification of antibodies from mouse ascites using Protein A magnetic immunomicrospheres

Having established a satisfactory protocol for the preparation of magnetic immunomicrospheres, we proceeded to investigate the feasibility of their use for antibody purification. Protein A, a cell-wall protein of staphylococcus aureus, is a single polypetide chain of MW 42,000 with four IgG Fc binding sites, two of which are active.[24] Protein A has a high specificity for immunoglobulins and is therefore suitable for the one-step capture of immunoglobulins.[25]

ProtA's ability to bind to the IgG molecule without interrupting its antigen-binding ability has been exploited for many immunological applications, including detection, purification, separation, and removal of host species and subclass-dependent antibodies. Efficient immobilization of ProtA is required for most of these bioapplications, and various methods of immobilization have been developed in recent years. However, most of them require chemical modifications of the supports. These modifications, leading to covalent binding of the ligand to the support, result in many cases in loss of activity of the ligand.[26] Therefore, the crucial test was whether these immunomicrospheres could be applied to the purification of antibody from a practical system and whether they would enable us to obtain a high purity product in this study.

It is evident from Figure 6 that this proved to be the case with mouse monoclonal antibodies IgG2a being successfully isolated from ascites extract in a one-step batch procedure, which took just 42 min to accomplish. It can be seen that the gel of lane 2 shows only two bands representing the heavy chain and light chain of the purified IgG2a. In the particular example shown, 10mg of magnetic microspheres were used to recover 0.22mg of IgG2a antibody. Similar results were obtained in other experiments independently. As a result, the IgG2a antibody purification efficiency was 22.0mg antibodies per gram of magnetic microspheres, which is comparable to the capacity of protein A affinity chromatography matrices.[27] So the magnetic separation process based on these ProtA-immobilized magnetic microspheres is effective and applicable to the immunoaffinity purification of antibodies. Further experimentation is now in progress to optimize the purification process and to access the potential of these magnetic microspheres in bioseparation.

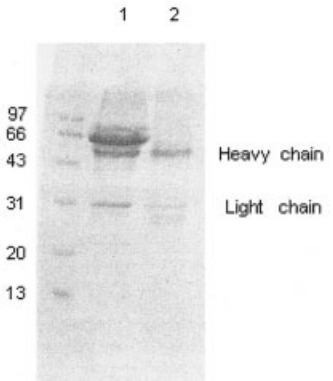


Figure 6 SDS-PAGE analysis of the IgG2a antibodies purified by the ProtA-immobilized magnetic microspheres (lane 2) and mouse ascites (lane 1). A Coomassie brilliant blue stained gel is shown. Marker proteins molecular mass (kDa) is shown on the left.

CONCLUSION

A novel and effective protocol for the preparation of superparamagnetic immunomicrospheres has been developed. Micro-size magnetic PMA-DVB spheres were prepared by a modified suspension polymerization. Then, the resulting magnetic microspheres were highly functionalized via ammonolysis reaction to form amino-modified magnetic microspheres. Compared with the conventional suspension copolymerization method, the magnetic microspheres made by this process have the advantages of smaller size (less than 8μm in diameter) with 80 percent of them in the size ranging from 1 to 5μm, as well as higher density of surface functional groups (—NH_2). They exhibit superparamagnetic characteristics with high saturation magnetization (σ_s = 13.8 emu/g). FT-IR spectroscopy confirmed the existence of reactive surface groups on the magnetic microspheres. Protein A was chose as affinity ligand and covalently immobilized onto the amino-modified magnetic microspheres by the glutaraldehyde method. These ProtA-immobilized magnetic immunomicrospheres were effective for the affinity bioseparation, which was demonstrated by the efficient immunoaffinity purification of antibodies IgG2a (22mg per gram of microspheres) from mouse ascites directly. These amino-modified magnetic PMA-DVB microspheres are an effective, inexpensive, and highly functionalized magnetic support. They have extensive potential application for magnetic bioaffinity separation, especially for cell isolation and sorting, immunoassay, and enzyme immobilization.

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References

1. Setchell, C. H. J Chem Technol Biotechnol 1985, 35B, 175.
2. Šafaøík, I.; Šafaøíková, M. J Chromatogr B 1999, 722, 33.
3. Hancock, J. P.; Kemshead, J. T. J Immun Methods 1993, 164, 51.
4. Sonti, S. V.; Bose, A. J Colloid Interface Sci 1995, 170, 575.
5. Shinkai, M.; Suzuki, M.; Iijima, S. Biotechnol Appl Biochem 1994, 21, 125.
6. Španová, A.; Rittich, B.; Horák, D. J Chromatogr A 2003, 1009, 215.
7. Ugelstad, J.; Ellingsen, T.; Berge, A. PCT. WO. Pat. 8303920, 1983.
8. Kondo, A.; Kamura, H.; Higashitani, K. Appl Microbiol Biotechnol 1994, 41, 99.
9. Ding, X.; Sun, Z.; Wang, G. Chinese J Polym Sci 1999, 17, 129.
10. Xie, G.; Zhang, Q. Y.; Luo, Z. P. J Appli Polym Sci 2003, 87, 1733.
11. Cocker, T. M.; Fee, C. J.; Evans, R. A. Biotechnol Bioeng 1997, 53, 79.
12. Lee, Y.; Rho, J.; Jung, B. J Appl Polym Sci 2003, 89 2058.
13. Guo, Z.; Bai, S.; Sun, Y. Enzyme Microb Technol 2003, 32, 776.
14. Guan, Y. P.; Liu, H. Z.; An, Z. T. CN. Pat. 98124516.1, 1998.
15. Kobayashi, H.; Matsunaga, T. J Colloid Interface Sci 1991, 141, 505.
16. Tanyolac, D.; Özdural, A. R. J Appl Polym Sci 2001, 80, 707.
17. Grulke, E. A. Suspension Polymerization; Wiley: New York, 1989.
18. Dowding, P. J.; Goodwin, J. W.; Vincent, B. Colloid Surface A 1998, 145, 263.
19. Ma, G. H.; Esumi, K. Polymer Interfaces and Emulsions; Marcel Dekker Inc.: New York, 1999.
20. Liu, X. Q.; Guan, Y. P.; Xing, J. M.; Ma, Z. Y.; Liu, H. Z. Chinese J Chem Eng 2003, 11, 731.
21. Lee, J.; Senna, M. Colloid Polym Sci 1995, 273, 76.
22. Liu, X. Y.; Ding, X. B.; Zheng, Z. H. J Appl Polym Sci 2003, 90, 1879.
23. Khng, H. P.; Cunliffe, D.; Davies, S. Biotechnol Bioeng 1998, 60, 419.
24. Surolia, A. Trends Biochem Sci 1982, 7, 318.
25. Tejeda-Mansir, A.; Espinoza, R.; Montesinos, R. M. Bioprocess Eng 1997, 17, 39.
26. Shpigel, E.; Goldlust, A.; Eshel, A. Biotechnol Appl Biochem 2000, 31, 197.
27. Füglistaller, P. J Immunol Methods 1989, 124, 171.